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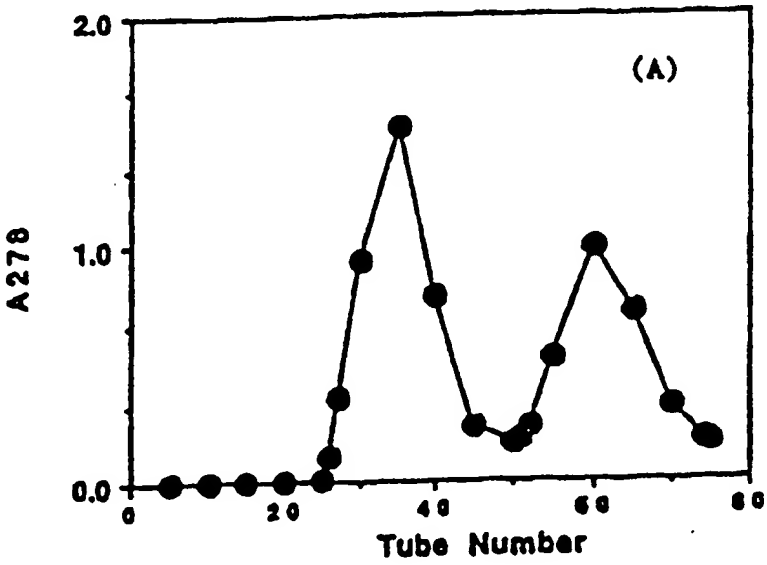
(54) Title: NOVEL PROTEINS WITHIN THE TYPE E BOTULINUM NEUROTOXIN COMPLEX

(57) Abstract

The invention features a polypeptide complex synthesized by bacteria of the genus *Clostridia* that contains the serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18kDa, respectively. The complex is useful in the treatment of diseases or conditions that are caused by excessive release of acetylcholine from presynaptic nerve terminals.



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NOVEL PROTEINS WITHIN THE TYPE E BOTULINUM NEUROTOXIN COMPLEX

Background of the Invention

The present invention relates to novel proteins that form a complex with the type E botulin neurotoxin produced by *Clostridium botulinum*.

Various strains of the bacterium *Clostridium*, including *C. botulinum*, *C. baratii*, and *C. butyricum*, synthesize different serotypes of the potent neurotoxin botulin, which causes a form of food poisoning known as botulism. *C. botulinum* synthesizes seven different serotypes, which are designated types A through G. Thes neurotoxins cause muscle paralysis by blocking the release of acetylcholine from cholinergic nerve endings (DasGupta et al., Biochemistry and Pharmacology of Botulinum and Tetanus Neurotoxins, *In* Perspective in Toxicology, Ed. by A. W. Bernheimer, Wiley, New York, NY, 1977).

Humans and other animals come into contact with botulinum neurotoxins most frequently by consuming food that is improperly stored in a way that permits growth of anaerobic bacteria. Typical foods tainted with botulin are low acid canned meats and vegetables, preserved meats and fish, and pasteurized processed cheese spreads (Fogeding, *In* Foodborne Microorganisms and Toxins: Developing Methodology, Eds. M.D. Pierson and N. Sterns, Marcel Dekker, Inc., New York, 1986; Kautter et al., J. Food Prot. 42:784-786, 1979). Another form of botulism, infant botulism, is thought to be caused by consumption of ubiquitous spores of *C. botulinum* along with food (Simpson, 1989, *In* Botulinum Neurotoxin and Tetanus Toxin, Academic Press, San Diego, CA). These spores may colonize the infant intestine, germinate, and produce the neurotoxin. Similarly, spores that gain access to deeply

wounded tissue may germinate and produce neurotoxin within the wound.

The nucleotide sequences of the genes encoding all of the different serotypes of the neurotoxin are known (Binz et al., J. Biol. Chem., 265:9153-9158, 1990; Campbell et al., J. Clin. Microbiol., 31:2255-2262, 1993; East et al., FEMS Microbiol. Lett., 96:225-230, 1992; Hauser et al., Nuc. Acids Re..., 18:4924, 1990; Whelan et al., Eur. J. Biochem., 204:657-667, 1992; and Whelan et al., Appl. Environm. Microbiol., 58:2345-2354, 1992). These genes are coordinately regulated with those encoding proteins that form a complex with the neurotoxin (Fujii et al., J. Gen Microbiol., 139:79-83, 1993; and Nukina et al., *In* Botulinum and Tetanus Neurotoxins, Ed. B.R. DasGupta, Plenum Press, New York, NY, 1993). The A and B type neurotoxins are associated with at least five other proteins, called "neurotoxin binding proteins," while the type E neurotoxin has been stated to be associated with one other protein (Sugii et al., Infect. Immunol., 12:1262-1270, 1975; Sakaguchi, Pharmac. Ther., 19:165-194, 1983; Schantz et al., Microbiol. Rev., 56:80-99, 1992; and Singh et al., J. Protein Chem., 14:7-18, 1995).

The proteins that associate with the type A neurotoxin are said to play a critical role in the food poisoning process by protecting the neurotoxin from the acids and proteolytic enzymes present in the gastrointestinal tract. The oral toxicity of isolated and purified type A neurotoxin is 43,000 fold less than the oral toxicity of the intact type A neurotoxin complex (Sakaguchi, Pharmac. Ther. 19:165-194, 1983). The proteins associated with other serotypes similarly "protect" the neurotoxin, but to a lesser degree.

Summary of the Invention

The invention is based on the discovery that, contrary to the conception in the field, the type E botulinum toxin exists in a complex that comprises the toxin and five neurotoxin associated proteins.

In general, the invention features a substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia*. The complex includes the serotype E botulinum neurotoxin and five neurotoxin associated polypeptides that have molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively. The 118 kDa polypeptide was known and sequenced previously, and has been referred to as a neurotoxin binding protein. The 80, 65, 40, and 18 kDa polypeptides in the complex are novel and, as described herein, have been partially sequenced. The 80 kDa polypeptide contains the amino acid sequence TNLKPYIIYD (SEQ ID NO:4), the 65 kDa polypeptide contains the amino acid sequence MQTTTLNWDT (SEQ ID NO:3), the 40 kDa polypeptide contains the amino acid sequence MRINTNINSM (SEQ ID NO:2), and the 18 kDa polypeptide contains the amino acid sequence MKQAFVFEFD (SEQ ID NO:1).

The invention also features a substantially pure antibody that specifically binds the type E neurotoxin complex, for example, by specifically binding to one or more, or all, of the five polypeptides in the complex. The antibody can be a substantially pure antibody (e.g., a monoclonal antibody, such as an IgA or IgG antibody) that specifically binds a polypeptide of the complex, or a fragment or derivative thereof. This antibody can be used to detect serotype E neurotoxin in a sample by contacting the sample with the antibody and detecting immune complexes formed between the antibody and the sample (e.g., by standard immunological techniques, such

as Western blotting or immunoprecipitation) as an indication of the presence of neurotoxin in the sample.

The discovery of these novel polypeptides provides the basis for a method of detecting the serotype E neurotoxin complex in a biological sample. The method includes obtaining an antibody that specifically binds a neurotoxin associated polypeptide, contacting the sample with the antibody, and detecting, if present, antibody-bound type E associated polypeptide. The presence of the antibody-bound polypeptide indicates the presence of serotype E neurotoxin in the sample, which may be a foodstuff or a biological sample, such as a gastrointestinal, blood, or tissue sample, obtained from a vertebrate animal.

As described herein for the first time, the novel 80 kDa type E neurotoxin associated polypeptide binds directly to the type E neurotoxin. Therefore, the 80 kDa neurotoxin associated polypeptide can also be used to detect the neurotoxin.

The discovery of the novel polypeptides of the invention also provides the basis for medicaments used in a method of treating a patient suffering from a disease or condition resulting from excessive acetylcholine release from presynaptic nerve terminals. The patient is treated by administering a therapeutically effective amount of a medicament including the serotype E neurotoxin complex. The excessive acetylcholine release can cause undesirable contraction of smooth or skeletal muscle cells, which can, in turn, cause spasmodic torticollis, essential tremor, spasmodic dysphonia, charley horse, strabismus, blepharospasm, oromandibular dystonia, spasms of the sphincters of the cardiovascular, gastrointestinal, or urinary systems, or tardive dyskinesia. The excessive acetylcholine release can also cause profuse sweating, lacrimation, or mucous secretion.

Alternatively, the patient may be a candidate for treatment according this method if suffering from spasticity that occurs secondary to brain ischemia, traumatic injury of the brain or spinal cord, tension headache, pain caused by sporting injuries, or pain caused by arthritic contractions.

In addition to treating the conditions described above, the novel polypeptides can be formulated as a vaccine and used to vaccinate an animal against serotype E neurotoxin by administering to the animal a serotype E neurotoxin complex and a physiologically acceptable carrier associated polypeptide.

By "purified antibody" is meant an antibody that is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, antibody.

By "specifically binds" is meant an antibody which recognizes and binds a polypeptide, but which does not substantially recognize and bind other molecules in a sample (e.g., a biological sample which includes type E neurotoxin associated polypeptides).

By "polypeptide" is meant any chain of two or more amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation), and thus includes peptides and proteins.

By "substantially pure polypeptide" is meant a polypeptide preparation that is at least 60% by weight the compound of interest, e.g., the serotype E neurotoxin associated polypeptides (or fragments or derivatives thereof). Preferably the preparation is at least 75%, more preferably at least 90% and most preferably at least 99%, by weight, the compound of interest. Purity can be measured by any appropriate method, e.g., column

chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Fig. 1 is an elution profile obtained by applying an extract of E-type producing *C. botulinum* to a DEAE-Sephadex A-50 ion-exchange column (A278 is absorbance at 278 nm).

Fig. 2 is a photograph of a polyacrylamide gel. Lane 1 was loaded with the material that eluted in the first peak of Fig. 1. Lane 2 was loaded with molecular weight standards. Lane 3 was loaded with material eluted from a G-200 column (see Fig. 5).

Fig. 3 is an elution profile obtained by applying the type E botulinum neurotoxin complex to a Sephadex G-100 column.

Fig. 4 is an elution profile obtained by applying the type E botulinum neurotoxin complex eluted from a Sephadex G-100 column to a Sephadex G-200 column.

Fig. 5 is an elution profile of the complex formed between type E botulinum neurotoxin and the 80 kDa component of the associated protein complex.

Fig. 6 is a photograph of an SDS-polyacrylamide gel. The material in the first and second peaks of the elution profile shown in Fig. 5 was electrophoresed in lane 1 and lane 2, respectively.

Fig. 7 is a three-dimensional plot generated by light scattering analysis of the type E botulinum complex. The first and second series of peaks were generated with 105 nm and 225 nm diameter particles, respectively.

Detailed Description

Contrary to the general understanding in the field, the type E botulinum toxin complex comprises the toxin, which has a molecular weight of about 150 kDa, and five (not one as previously believed) polypeptides that form a complex with the neurotoxin. These five polypeptides have molecular weighs of approximately 118, 80, 65, 40, and 18 kDa, and can be used individually, or in combination with the neurotoxin in the complex, to generate novel antibodies and vaccines.

Bacterial Cell Culture

Generally, to obtain botulinum toxin in large amounts, complexed media containing combinations of meat hydrolysate, casein hydrolysate, yeast autolysate, yeast extract, and glucose supplemented with one or more reducing agents are used (Sakaguchi, Pharmac. Ther., 19:165-194, 1983). Vegetables autoclaved in saline also provide an excellent culture medium, supporting toxin

production by type A- and type B-producing bacteria to a similar extent as laboratory media. Glucose must be added for type E- and type F-producing bacteria to grow in boiled vegetables (Sugii et al., J. Food Safety, 1:53-65, 1977). The optimum temperature for toxin production by *C. botulinum* is generally regarded as 20-35°C.

Type E *C. botulinum* Produces a Complex Including Five Neurotoxin Associated Proteins

For this series of experiments, *C. botulinum* type E (available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, 20852 (U.S.A.); Type E *Clostridium botulinum* Accession Nos. 9564, 17786, 17852, 17854, and 17855) was grown for 4 days in 15 ml cooked meat medium. Stock cultures were prepared according to standard methods and stored at -20°C.

The stock culture was activated at 30°C for approximately 25 hours and then transferred to a growth medium containing 2.0% Trypticase-peptone, 1.0% glucose, 0.025% sodium thioglycolate (BBL Microbiology Systems, Cockeysville, MD), and 0.5% yeast extract (Difco) adjusted to pH 6.5. When large culture volumes (8 liters) were used, a 12% glucose solution was autoclaved and added to the broth, which was separately prepared and then autoclaved for 1 hour. The culture was incubated for 60-65 hours, and cells were collected by centrifugation. An extract from the cells was prepared at 20°C by stirring with 0.2 M phosphate buffer (pH 6.0). The resulting suspension was saturated with $(NH_4)_2SO_4$; 39 g/ml) and stored at 4°C.

DEAE-Sephadex Chromatography

The crude extract described above was precipitated and redissolved in 35 ml of 0.05 M sodium phosphate buffer (pH 5.5). The resulting solution was clarified by centrifugation and chromatographed on a DEAE-Sephadex A-50 ion-exchange column (Pharmacia). The sample was eluted from the column with 0.05 M sodium citrate at

pH 5.5. It is important that the pH of the buffer is maintained at 5.5. The first protein peak (Fig. 1) was pooled as type E complex.

In contrast to previous reports (Sugii et al., Infect. Immunol., 12:1262-1270, 1975; Sakaguchi, Pharmac. Ther., 19:165-194, 1983; Schantz et al., Microbiol. Rev., 56:80-99, 1992; Singh et al., J. Protein Chem., 14:7-18, 1995), a total of five different proteins were found in the complex in addition to the 150 kDa type E botulinum neurotoxin. Specifically, the material constituting the first peak of the elution profile described above (and shown in Fig. 1) was analyzed by SDS-polyacrylamide gel electrophoresis. Six proteins, having molecular weights of approximately 150 (the neurotoxin), 118, 80, 65, 40, and 18 kDa, were apparent (Fig. 2).

Size Exclusion Chromatography

To further confirm the nature of the type E complex, the proteins were analyzed on size exclusion chromatographic columns. The type E complex eluted from the DEAE Sephadex A-50 column was concentrated to 5 mg/ml and applied to a Sephadex G-100 column (1.8 x 92 cm or 2.6 x 82 cm, 0.05 M sodium citrate buffer, pH 5.5). The resulting elution profile revealed one peak in the void volume (Fig. 3). Three of the proteins present are clearly less than the exclusion limit of the column and thus, should elute separately from the void volume. Since this did not occur, and all six proteins continued to elute in one peak, it was concluded that the proteins are bound together in a complex. A similar result was obtained following chromatography on a Sephadex G-200 column (Fig. 4), further confirming that the six proteins form a single complex.

One of the neurotoxin associated proteins in the type E complex, the 80 kDa protein, was purified and studied for its ability to re-form a complex with pure

type E neurotoxin. After combining the 150 kDa neurotoxin and the 80 kDa associated protein, the elution profile obtained from a Sephadex G-200 column revealed a major peak containing both the 80 kDa protein and the type E neurotoxin, and a minor peak containing excess uncomplexed 80 kDa protein (Fig. 5). The material eluted in each of the two peaks was electrophoresed on an SDS-polyacrylamide gel (Fig. 6), which confirmed the content of the protein(s) in each peak.

The 80 kDa Neurotoxin Associated Protein Specifically Binds Type E Neurotoxin

A kinetic binding study performed with an optical fiber-based biosensor revealed that the type E neurotoxin could bind directly to the 80 kDa type E neurotoxin associated protein, rather than associate indirectly with the neurotoxin via another polypeptide in the complex. The 80 kDa polypeptide was tested for its ability to bind directly to the neurotoxin at pH 7.5 and at pH 5.7. The type E botulinum neurotoxin was first immobilized, and purified neurotoxin binding polypeptide (NBP; 80 kDa) was labeled with TRITC (Tetramethylrhodamine-isothiocyante; Molecular Probes, Eugene, OR) as described in Ogert et al., Anal. Biochem., 205:306-312, 1992, except that unreacted TRITC was removed by dialysis, rather than gel filtration. The binding experiments were carried out by blocking the exposed sites on the optical fiber with 2% BSA (at room temperature) and incubating them with TRITC-labeled 80 kDa polypeptide (5 mg/ml) that had been equilibrated with phosphate buffered saline (PBS; at pH 5.7 or pH 7.5). The initial rate of binding was calculated based on the signal increase within the first 60 seconds.

Subsequent polypeptide binding rates at pH 7.5 and 5.7 were 4.01 and 8.42 uV/minute, respectively, suggesting that the interaction between the neurotoxin and the 80 kDa polypeptide is significant at pH 7.5, and

considerably higher at pH 5.7. Therefore, the 80 kDa polypeptide could play a role in protecting the type E neurotoxin from the acidic conditions present in the gastrointestinal tract. These results are consistent with the known behavior of the botulinum neurotoxin complex, which dissociates at alkaline pH levels. Thus, the associated binding polypeptides can be used as a specific binding partner to "capture," and thereby detect, the neurotoxin. This method would effectively detect the neurotoxin wherever it exists, to at least some degree, free from the complex or, at least, free from the 80 kDa neurotoxin binding protein.

Sequence Analysis of Proteins
in the Type E Neurotoxin Complex

Partial amino acid sequences of the novel polypeptides in the serotype E neurotoxin complex were obtained as follows. Approximately 10 picomoles of the purified type E neurotoxin complex were dissolved in a buffer consisting of 0.5 M sucrose, 15% SDS (sodium dodecyl sulfate), 312.5 mM Tris, and 10 mM EDTA, and electrophoresed on a 12.5% SDS-acrylamide gel using a Mini-PROTEAN II™ electrophoresis cell (Bio-Rad Laboratories, Hercules, CA). The electrophoresis was performed in running buffer (2 g/L Tris base, 14.4 g/L glycine, 1 g/L SDS and 0.1 mM sodium thioglycolate, pH 8.3) under a constant voltage (200 V). The protein was then electrotransferred from the gel to a PVDF membrane in a Twobin buffer (25 mM Tris, 192 mM glycine and 20% methanol) using a Mini Trans-Blot electrophoretic transfer cell™ (Bio-Rad Laboratories, hercules, CA). The transfer was carried out overnight at 60 volts in an ice bath. To visualize the protein bands, the membrane was stained with 0.025% Coomassie Blue R250 in 40% methanol and destained with 50% methanol. The proteins bound to the PVDF membranes were sequenced at Baylor College of

M dicine (Houston, TX) using Applied Biosystem Model 473A protein sequencer™ (Foster City, CA).

The following peptide sequences were obtained: (1) MKQAFVFEFD (SEQ ID NO:1), from the 18 kDa protein; (2) MRINTNINSM (SEQ ID NO:2), from the 40 kDa protein; (3) MQTTTLNWDT (SEQ ID NO:3), from the 65 kDa protein; and (4) TNLKPYIIYD (SEQ ID NO:4), from the 80 kDa protein. These sequences were compared with those of known proteins associated with neurotoxin types A, B, and C. This analysis failed to reveal any regions of homology with the type A associated proteins of *C. botulinum*.

Analysis of Type E Neurotoxin Complex by Light Scattering

To characterize the type E neurotoxin complex as a whole, light scattering experiments were performed on material purified by DEAE-Sephadex A-50 chromatography (1.5 mg/ml). Analysis was performed on a Malvern 4700 PCS Autosizer System (Malvern Instruments Inc.) equipped with an eight-bit, 136 channel correlator capable of variable time expansion. The laser light source was model INNOVA 70-5 argon laser (Coherent, CA). A 514.5 nm line was employed in single operation mode with 1.0 watt power output.

Initial results from light scattering experiments suggest that the complex exists in two forms, as 600 and 2000 kDa molecular weight species (Fig. 7). The combined molecular weight of the proteins in the type E neurotoxin complex observed on polyacrylamide gels is 468 kDa. The difference between these two predicted sizes could be due either to variation in the folding of the complex or to the existence of oligomeric forms of some of the proteins in the complex.

Production of Antibodies Against the Type E Neurotoxin Associated Proteins

The novel proteins described herein are indicators of the presence of type E neurotoxin. The type A neurotoxin remains associated with its protein complex both in bacterial culture medium and in natural cases of food poisoning (Sakaguchi, Pharmac. Ther., 19:165-194, 1983). Given this evidence, it is likely that the 118 kDa binding protein and the other four, lower molecular weight members of the type E complex also remain associated with the cognate toxin *in vitro* and *in vivo*. In addition, neurotoxin associated proteins have been shown to be more immunogenic than the neurotoxin itself (Singh et al., 1996, Toxicon 34:267-275).

A variety of standard methods can be used to generate antibodies against the type E neurotoxin associated proteins. For example, the type E neurotoxin associated proteins, either individually or in their complex forms, can be administered to an animal, such as a rabbit, to induce the production of polyclonal antibodies. Alternatively, antigenic fragments of the individual polypeptides may be used to generate polyclonal antibodies.

In addition, antibodies according to the invention can be monoclonal antibodies generated by using either individual serotype E associated polypeptides or the intact type E complex. Such monoclonal antibodies can be prepared using standard hybridoma technology (see; e.g., Kohler et al., Nature, 256:495, 1975; Kohler et al., Eur. J. Immunol., 6:292, and 6:511, 1976; Hammerling et al., *In* Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981; Kruisbeck et al., Hornbeck et al., and Yokoyama, *In* Current Protocols in Immunology, Vol. I, New York, John Wiley & Sons, Inc., 1994).

The binding specificity and activity of purified anti-type E complex antibodies can be confirmed by

testing their ability to interfere with the biological activity of the neurotoxin and/or the complex. This can be tested by adding the antibodies to any number of standard *in vitro* assays in which the release of acetylcholine from presynaptic nerve terminals can be monitored. These assays include preparations of different neuromuscular junctions, such as the mouse phrenic nerve-hemidiaphragm, the mouse plantar nerve-lumbrical muscle, and chick ciliary ganglion-iris muscle preparations (Bandyopadhyay et al., J. Biol. Chem., 262:2660-2663, 1987); Bittner et al., J. Biol. Chem., 264:10354-10360, 1989; Clark et al., J. Neurosci. Methods, 19:285-295, 1987; and Lomneth et al., Neurosci. Lett., 113:211-216, 1990). The binding specificity and activity of any given antibody is tested by determining whether that antibody effectively blocks the action of type E neurotoxin complex applied at the neuromuscular junction.

Antibody-Based Detection Systems for Type E Neurotoxin Associated Proteins

Antibodies can be used to detect the type E neurotoxin complex using various standard methods. For example, the antibodies can be used with a fiber optic-based biosensor, as described above, which uses an evanescent wave from a tapered optical fiber for signal discrimination. This antibody-based "sandwich" immunoassay detection system can detect botulinum toxin much more quickly than any method currently available, but other immunoassay methods can be used. The actual signal collection period with the biosensor is less than one minute. Detection is accomplished using a two-step sandwich immunoassay. Antibody-bound optical fibers are incubated in a solution of type E complex, and a signal is generated when the fiber-bound complex binds a fluorescently labeled antibody (see, Ogert et al., Anal. Biochem., 205:306-312, 1992; and Singh et al., *In* Natural

Toxins II, Ed. B.R. Singh and A. Tu, Plenum Press, pp. 498-508, 1996).

One of the problems historically associated with sandwich immunoassays is that the first antibody (here, the antibody bound to the optical fiber) and the second antibody (here, the antibody added to detect the fiber-bound complex), compete for the same epitope on the neurotoxin. To circumvent this problem, two antibodies can be used. The first against one portion of the neurotoxin or one member of the type E complex, which will be attached to the fiber, and a second against either a second portion of the neurotoxin or a second member of the polypeptide complex, which would specifically recognize the fiber-bound complex.

Preparation and Administration of A Serotype E Neurotoxin Vaccine

The invention also includes a vaccine composition containing, in addition to type E neurotoxin and neurotoxin associated polypeptides (or immunogenic fragments or derivatives thereof), a pharmaceutically acceptable diluent or carrier, such as phosphate buffered saline or a bicarbonate solution (e.g., 0.24 M $NaHCO_3$). The carrier and diluents used in the invention are selected on the basis of the mode and route of administration, and standard pharmaceutical practice. Suitable pharmaceutical carriers and diluents, as well as pharmaceutical necessities for their use, are described in Remington's Pharmaceutical Sciences. An adjuvant, e.g., a cholera toxin, *Escherichia coli* heat-labile enterotoxin (LT), or a fragment or derivative thereof having adjuvant activity, may also be included in the vaccine composition of the invention.

Skilled artisans can obtain further guidance in the preparation of a vaccine for type E neurotoxin complex in Singh et al. (1990, Toxicon 27:403-410). Briefly, approximately 1.5 mg of the type E complex is

added to approximately 10 ml of 0.05 M sodium citrate buffer (pH 5.5) and dialyzed against 0.39% formaldehyde at 30°C for seven days. The formaldehyde-containing buffer is replaced every day with fresh buffer solution. The detoxified neurotoxin (toxoid or vaccine) is dialyzed against 0.1 M sodium phosphate buffer (pH 7.4) without formaldehyde for two days with several changes of buffer.

The amount of vaccine administered will depend, for example, on the particular vaccine antigen, whether an adjuvant is co-administered with the antigen, the type of adjuvant co-administered, the mode and frequency of administration, and the desired effect (e.g., protection or treatment), as can be determined by one skilled in the art. In general, the vaccine antigens of the invention are administered in amounts ranging between, e.g., 1 μg and 100 mg. If adjuvants are administered with the vaccines, amounts ranging between, e.g., 1 ng and 1 mg can be used. Administration is repeated as necessary, as can be determined by one skilled in the art. For example, a priming dose can be followed by three booster doses at weekly intervals.

USE

The novel proteins discovered in the type E botulinum neurotoxin complex can be used in a number of ways. They can be used, for example, to help prevent botulism. First, they can be used to generate antibodies, as described above, that can be used to detect the presence of the toxin in foods. Second, they can be used to generate vaccines for immunization. In addition, if a patient is exposed to the neurotoxin, these proteins provide the means, e.g., through antibody-based or direct binding detection systems, for rapid and reliable diagnosis. The proteins, in their naturally occurring complex with the type E neurotoxin, are also

useful in treating diseases associated with excessive release of acetylcholine from cholinergic nerve terminals.

Advantages

Administration, as described below, of the intact type E neurotoxin complex is superior to administration of the neurotoxin alone in that the complex is longer-lasting. This feature minimizes the frequency of administration and thus, reduces any risk, discomfort, or inconvenience that the patient may experience.

The type E complex is a superior therapeutic agent, relative to the other botulinum serotypes, because the activity of the type E neurotoxin can be enhanced 100-fold by treatment with trypsin, which breaks the bonds between the two polypeptide chains that constitute the neurotoxin. Therefore, application of the type E neurotoxin complex can be controlled by trypsinization, in a way that allows graded release of the neurotoxin from the complex. This unique mechanism provides more controlled and longer-lasting effects than would otherwise be possible.

Disease Treatment with Type E Neurotoxin Complex

Any disease or discomfort associated with an exaggerated release of acetylcholine from a presynaptic nerve terminal can be treated with the type E botulinum neurotoxin complex described herein. These diseases are associated with either smooth or skeletal muscle spasms, such as spasmodic torticollis, essential tremor, spasmodic dysphonia, charley horse, strabismus, blepharospasm, oromandibular dystonia, spasms of the sphincters of the cardiovascular, gastrointestinal, or urinary systems, and tardive dyskinesia, which may result from treatment with an anti-psychotic drug such as THORAZINE® or HALDOL®.

For example, an adult male patient suffering from tardive dyskinesia resulting from treatment with an antipsychotic drug can be treated with 50-200 units (defined below) of Botulinum type E complex by direct injection into the facial muscles. Within three days, the symptoms of tardive dyskinesia, i.e., orofacial dyskinesia, athetosis, dystonia, chorea, tics and facial grimacing are markedly reduced.

Spasticity that occurs secondary to brain ischemia, or traumatic injury of the brain or spinal cord, are similarly amenable to treatment.

In instances where the postsynaptic target is a gland, nerve plexus, or ganglion, rather than a muscle, the type E complex can be administered to control profuse sweating, lacrimation, and mucous secretion. For example, an adult male patient with excessive unilateral sweating can be treated by administering 0.01 to 50 units of type E botulinum complex to the gland nerve plexus, ganglion, spinal cord, or central nervous system. Preferably, the nerve plexus or ganglion that malfunctions to produce the excessive sweating is treated directly. Administration of type E neurotoxin complex to the spinal cord or brain, while feasible, may cause general weakness.

Other conditions that can be treated include tension headache and pain caused by sporting injuries or arthritic contractions. If necessary, overactive muscles can be identified with electromyography (EMG).

Administration of the
Type E Neurotoxin Complex

The dose of type E neurotoxin complex administered to a patient will depend generally upon the severity of the condition, the age, weight, sex, and general health of the patient, and the potency of the toxin, which is expressed as a multiple of the LD_{50} value for the mouse.

The dosages used in human therapeutic applications are roughly proportional to the mass of muscle in need of treatment. Typically, the dose administered to the patient may be from about 0.01 to about 1,000 units, for example, about 500 units. A unit is defined as the amount of type E neurotoxin (or type E neurotoxin complex) that kills 50% of a group of mice (typically a group of 18-20 female mice that weigh on average 20 grams). The dosage is adjusted, either in quantity or frequency, to achieve sufficient reduction in acetylcholine release to afford relief from the symptoms of the disease or condition being treated.

Physicians, pharmacologists, and other skilled artisans are able to determine the most therapeutically effective treatment regimen, which will vary from patient to patient. The potency of botulinum toxin and its duration of action means that doses are administered on an infrequent basis. Skilled artisans are also aware that the treatment regimen must be commensurate with questions of safety and the effects produced by the toxin.

Typically, the type E neurotoxin complex is suspended in a physiologically acceptable solution, such as normal saline, and is administered by an intramuscular injection. Prior to injection, careful consideration is given to the anatomy of the muscle group, in an attempt to inject the toxin complex into the area with the highest concentration of neuromuscular junctions. If the muscle mass is not very great, the injection can be performed with extremely fine, hollow, teflon-coated needles and guided by electromyography. The position of the needle in the muscle should be confirmed prior to injection of the toxin, and general anesthesia, local anesthesia, or other sedation may be used at the discretion of the attending physician, according to the

age and particular needs of a given patient and the number of sites to be injected.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: University of Massachusetts Dartmouth

(ii) TITLE OF INVENTION: NOVEL PROTEINS WITHIN THE TYPE E BOTULINUM NEUROTOXIN COMPLEX

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Fish & Richardson P.C.
(B) STREET: 225 Franklin Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02110-2804

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/US96/-----
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Fasse, Peter J.
(B) REGISTRATION NUMBER: 99,999
(C) REFERENCE/DOCKET NUMBER: 08387/002W01

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617/542-5070
(B) TELEFAX: 617/542-8906
(C) TELEX: 200154

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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1 5 10

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: not relevant
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Thr Asn Leu Lys Pro Tyr Ile Ile Tyr Asp
1 5 10

What is claimed is:

1. A method of detecting a serotype E neurotoxin complex in a biological sample, said method comprising:

(a) obtaining an antibody that specifically binds a polypeptide of a substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia*, said complex comprising serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively,

(b) contacting said sample with said antibody, and

(c) detecting antibody-bound polypeptide, if any, in said sample, the presence of said antibody-bound polypeptide indicating the presence of serotype E neurotoxin in said sample.

2. A method of detecting a serotype E neurotoxin in a biological sample, said method comprising:

(a) obtaining a polypeptide that specifically binds a neurotoxin polypeptide from a substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia*, said complex comprising serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively,

(b) contacting said sample with said polypeptide, and

(c) detecting polypeptide-bound neurotoxin, if any, in said sample, the presence of said polypeptide-bound neurotoxin indicating the presence of serotype E neurotoxin in said sample.

3. The method of any one of claims 1 or 2, wherein said sample is a foodstuff.

4. The method of any one of claims 1 or 2, wherein said sample is a gastrointestinal, blood, or tissue sample obtained from a vertebrate animal.

5. A substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia* for use in treating a disease resulting from excessive acetylcholine release from presynaptic nerve terminals, said complex comprising serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively.

6. The use of a substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia* for the manufacture of a medicament for treating a disease resulting from excessive acetylcholine release from presynaptic nerve terminals, said complex comprising serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively.

7. The polypeptide of any one of claims 5 or 6, wherein said excessive acetylcholine release causes undesirable contraction of smooth or skeletal muscle cells.

8. The polypeptide of claim 7, wherein said undesirable muscular contraction causes spasmodic torticollis, essential tremor, spasmodic dysphonia, charley horse, strabismus, blepharospasm, oromandibular dystonia, spasms of the sphincters of the cardiovascular, gastrointestinal, or urinary systems, or tardive dyskinesia.

9. The polypeptide of any one of claims 5 or 6, wherein said excessive acetylcholine release causes profuse sweating, lacrimation, or mucous secretion.

10. A substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia* for use in treating spasticity occurring secondary to brain ischemia, traumatic injury of the brain or spinal cord, a tension headache, or pain caused by sporting injuries or arthritic contractions, said complex comprising serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively.

11. The use of a substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia* for the manufacture of a medicament for treating spasticity occurring secondary to brain ischemia, traumatic injury of the brain or spinal cord, a tension headache, or pain caused by sporting injuries or arthritic contractions, said complex comprising serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively.

12. A vaccine composition comprising a substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia* and a physiologically acceptable carrier, said complex comprising serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively.

13. A substantially pure polypeptide complex synthesized by bacteria of the genus *Clostridia*, said

complex comprising serotype E botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively.

14. A complex of claim 13, wherein one of said polypeptides has a molecular weight of approximately 80 kDa and comprises the amino acid sequence TNLKPYIIYD (SEQ ID NO:4).

15. A complex of claim 13, wherein one of said polypeptides has a molecular weight of approximately 65 kDa and comprises the amino acid sequence MQTTTLNWDT (SEQ ID NO:3).

16. A complex of claim 13, wherein one of said polypeptides has a molecular weight of approximately 40 kDa and comprises the amino acid sequence MRINTNINSM (SEQ ID NO:2).

17. A complex of claim 13, wherein one of said polypeptides has a molecular weight of approximately 18 kDa and comprises the amino acid sequence MKQAFVFEFD (SEQ ID NO:1).

18. A substantially pure antibody, said antibody specifically binding the complex of claim 13.

19. A substantially pure antibody, said antibody specifically binding to one of said five polypeptides in said complex of claim 13.

20. A substantially pure polypeptide isolated from a polypeptide complex synthesized by bacteria of the genus *Clostridia*, said complex comprising serotype E

botulinum neurotoxin and five neurotoxin associated polypeptides having molecular weights of about 118, 80, 65, 40, and 18 kDa, respectively.

FIG. 1

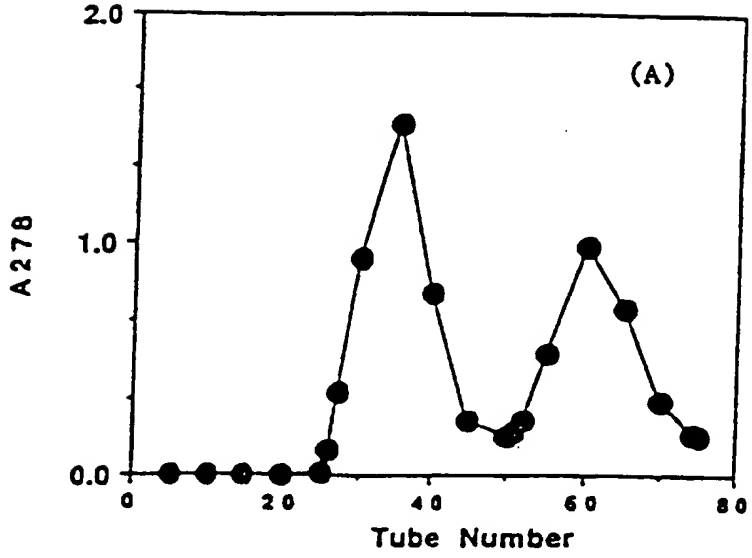


FIG. 2

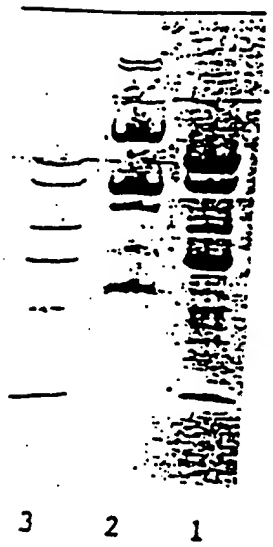


FIG. 3

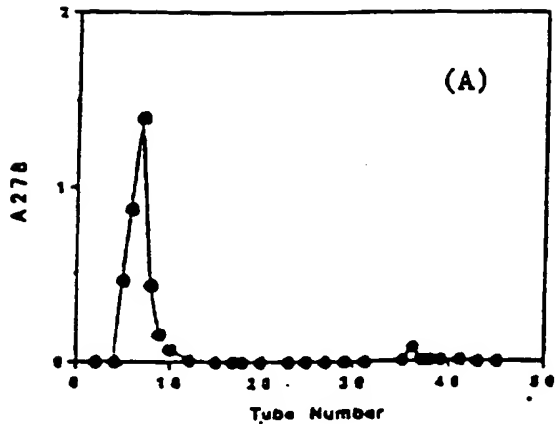


FIG. 4

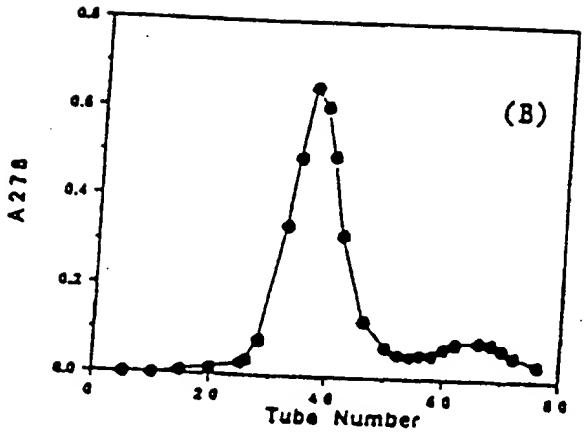


FIG. 5

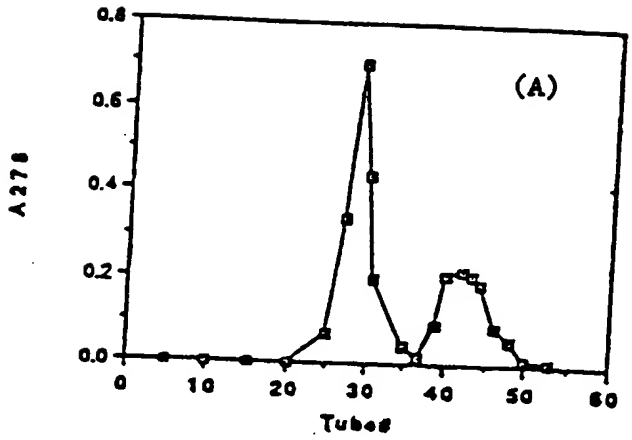
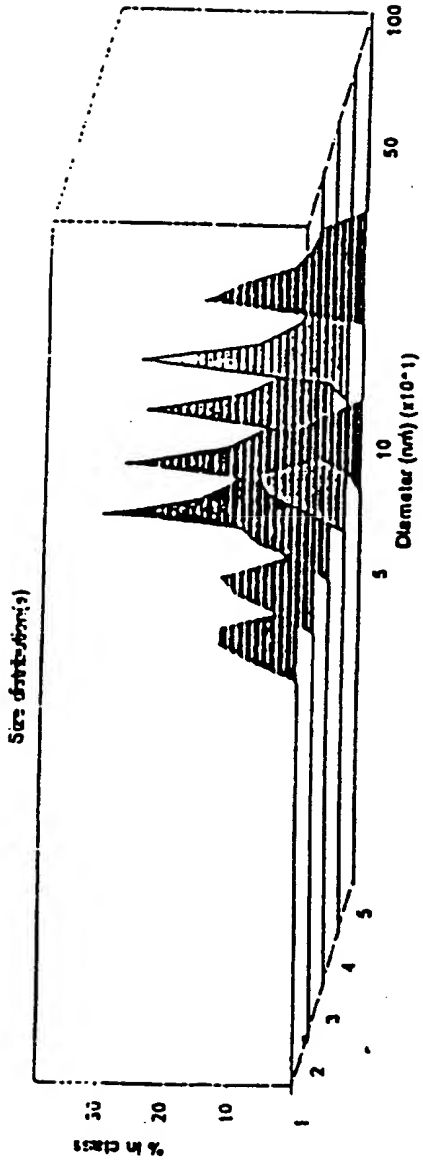


FIG. 6

FIG. 7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Diameter (nm) (x10^-1)

INTERNATIONAL SEARCH REPORT

Int tional application No.
PCT/US96/11383

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/53

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.32; 424/141.1,142.1, 150.1, 152.1, 158.1, 167.1, 172.1, 190.1, 247.1; 436/501, 536, 543, 547, 20; 530/300, 412

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS

search terms: boulinum, type E, neurotoxin, characterize or analysis

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	SINGH et al. Physicochemical and Immunological Characterization of the Type E Botulinum Neurotoxin Binding Protein Purified from Clostridium Botulinum. Journal of Protein Chemistry. 1995. Vol. 14, No. 1, pages 7-18. See entire document.	5, 7-10, 13-20 ---------- 1-4, 6, 11-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 OCTOBER 1996	13 NOV 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer RACHEL FREED Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

In ational application No.
PCT/US96/11383

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.1, 7.32; 424/141.1,142.1, 150.1, 152.1, 158.1, 167.1, 172.1, 190.1, 247.1; 436/501, 536, 543, 547, 20; 530/300, 412

FIG. 1

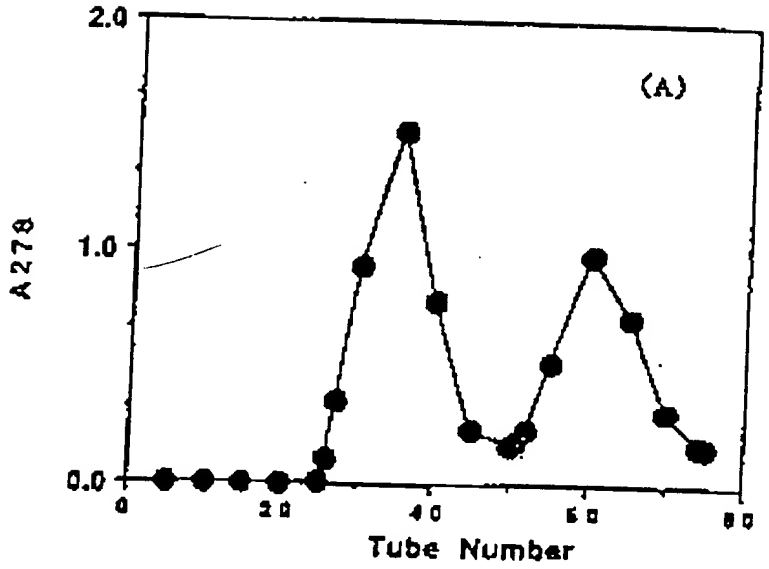

FIG. 2

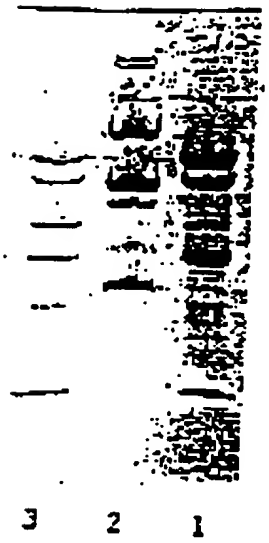

FIG. 3

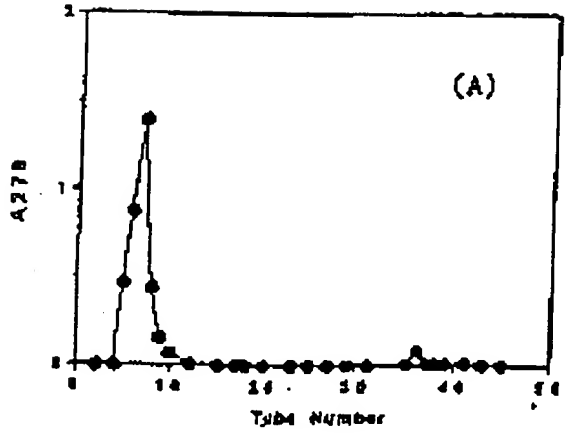

FIG. 4

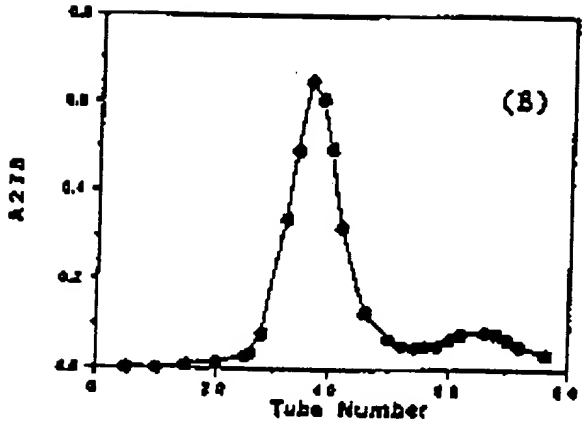

FIG. 5

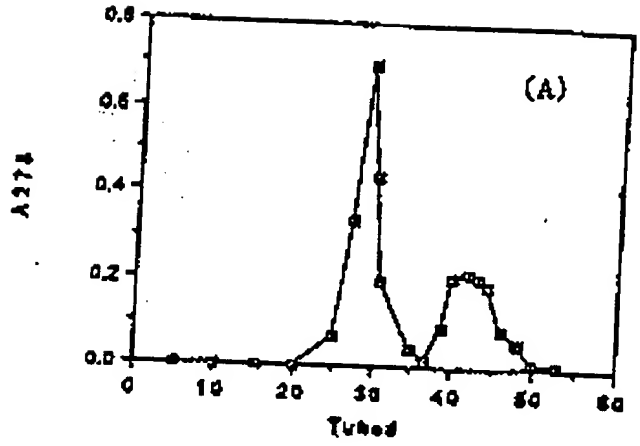

FIG. 6

FIG. 7